

# Glucose Restriction Inhibits Skeletal Myoblast Differentiation by Activating SIRT1 through AMPK-Mediated Regulation of Nampt

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## SUMMARY

It is intuitive to speculate that nutrient availability may influence differentiation of mammalian cells. Nonetheless, a comprehensive complement of the molecular determinants involved in this process has not been elucidated yet. Here, we have investigated how nutrients (glucose) affect skeletal myogenesis. Glucose restriction (GR) impaired differentiation of skeletal myoblasts and was associated with activation of the AMP-activated protein kinase (AMPK). Activated AMPK was required to promote GR-induced transcription of the NAD<sup>+</sup> biosynthetic enzyme Nampt. Indeed, GR augmented the Nampt activity, which consequently modified the intracellular [NAD<sup>+</sup>]:[NADH] ratio and nicotinamide levels, and mediated inhibition of skeletal myogenesis. Skeletal myoblasts derived from *SIRT1*<sup>+/-</sup> heterozygous mice were resistant to the effects of either GR or AMPK activation. These experiments reveal that AMPK, Nampt, and SIRT1 are the molecular components of a functional signaling pathway that allows skeletal muscle cells to sense and react to nutrient availability.

## INTRODUCTION

The execution of essential and specialized functions relies on the cell's ability to sense and adapt to the environment, with nutrient availability being one of the most critical variables. The nematode *C. elegans* reacts to overcrowding and unfavorable nutrient conditions by entering the dauer diapause, a nonfeeding, resistant to oxidative stress, and long-lived larval developmental stage (Cassada and Russell, 1975). Moreover, decreasing calorie intake is the sole intervention that successfully increases life span across species (Masoro, 2006). A mechanistic understanding of these phenomena requires the identification of the molecules that mediate the cellular response to nutrients.

Although their role on lifespan is still a matter of intense debate (Chen and Guarente, 2007; Kaeberlein and Powers, 2007; Longo

and Kennedy, 2006; Michan and Sinclair, 2007), it is well established that the sirtuins—a family of evolutionarily conserved deacetylases—play important roles in numerous physiological processes (Haigis and Guarente, 2006). By deacetylating cofactors such as PCAF (Fulco et al., 2003; Brunet et al., 2004), p300 (Motta et al., 2004; Brunet et al., 2004), PGC-1 $\alpha$  (Nemoto et al., 2005; Rodgers et al., 2005), and numerous transcriptional activators, the NAD<sup>+</sup>-dependent deacetylase sirtuin SIRT1 controls critical functions of mammalian cell physiology including stress resistance (Vaziri et al., 2001; Luo et al., 2001; Cohen et al., 2004), replicative senescence (Chua et al., 2005), aging, and differentiation (Blander and Guarente, 2004). Differentiation of skeletal muscle cells (Fulco et al., 2003) and adipocytes (Picard et al., 2004), angiogenesis (Potente et al., 2007), survival of neurons (Araki et al., 2004) and pancreatic  $\beta$  cells (Kitamura et al., 2005), insulin secretion (Bordone et al., 2006; Moynihan et al., 2005), lipid (Li et al., 2007) and liver metabolism (Rodgers et al., 2005), and increased physical activity during calorie restriction (Chen et al., 2005) are all regulated by SIRT1.

In contrast to class I-II histone deacetylases (HDACs), the enzymatic activity of SIRT1 is modulated by physiological cofactors and inhibitors. NAD<sup>+</sup> is an obligate cosubstrate (Imai et al., 2000), whereas NADH (Lin et al., 2004) and nicotinamide (NAM) are inhibitors of SIRT1 (Bitterman et al., 2002). The central role of the NAD<sup>+</sup> salvage pathway in regulating the enzymatic activity of Sir2—the SIRT1 yeast ortholog—is illustrated by the observation that the nicotinamidase PNC1—the yeast functional equivalent of mammalian NAM phosphoribosyltransferase (Nampt; also known as PBEF or visfatin)—is both necessary and sufficient for life span extension caused by calorie restriction and low-intensity stress in a Sir2-dependent fashion (Anderson et al., 2003). Moreover, Nampt retards senescence of cultured human cells (van der Veer et al., 2007). Overexpression of exogenous Nampt regulates the transcriptional activity of a transiently transfected Gal-SIRT1 fusion protein in mammalian cells (Revollo et al., 2004). Nampt was recently identified as a stress- and nutrient-responsive gene that increases mitochondrial NAD<sup>+</sup> levels and promotes survival during genotoxic stress via the mitochondrial sirtuins SIRT3 and SIRT4 (Yang et al., 2007). Even though it remains unclear as to what the relative contribution of increased [NAD<sup>+</sup>]:[NADH] ratio versus reduced NAM is,

overall, these findings are consistent with the suggestion that modifications in  $\text{NAD}^+$  and NAM levels are likely to be the most important regulators of sirtuin activity (Grubisha et al., 2005). Despite a wealth of information on the molecules and mechanisms that mediate the effects of SIRT1 on several biological processes (Michan and Sinclair, 2007), the identification and mechanistic elucidation of the signals that activate the  $\text{NAD}^+$  salvage pathway and, as a consequence, regulate the deacetylase activity of SIRT1 and of other sirtuins in response to nutrient availability and oxidative stress in mammalian cells remains to be fully understood. Within this context, an attractive candidate is the AMP-activated protein kinase (AMPK). AMPK activation is observed in fasting and calorie-restricted animals and it has been proposed as a one of the several mechanisms involved in regulating mammal longevity (McCarty, 2004). In agreement with this hypothesis—and, similarly, to Sir2—extra copies of AMPK can extend life span in *C. elegans* (Apfeld et al., 2004) and mediate the effects of dietary restriction on longevity through the FOXO transcription factors (Greer et al., 2007a). Finally, the SIRT1 agonist resveratrol—shown to augment survival of mice on a high-calorie diet (Baur et al., 2006) and improve mitochondrial function (Lagouge et al., 2006)—induces phosphorylation and activation of AMPK (Baur et al., 2006; Dasgupta and Milbrandt, 2007).

Skeletal muscle cell differentiation is accompanied by modifications of the  $[\text{NAD}^+]:[\text{NADH}]$  ratio that exerts regulatory functions on SIRT1 (Sartorelli and Caretti, 2005). A decrease of the  $[\text{NAD}^+]:[\text{NADH}]$  ratio coincides with skeletal myogenesis, whereas an increase inhibits it (Fulco et al., 2003). The regulatory function of the  $[\text{NAD}^+]:[\text{NADH}]$  ratio offers the opportunity to investigate whether a link exists between the mechanisms that preside to differentiation and those that mediate the response to nutrient availability in skeletal muscle cells. Here, we report that GR causes AMPK activation and prevents proper differentiation of mouse skeletal muscle cells. Activated AMPK is required to induce Nampt transcription, thus increasing intracellular  $[\text{NAD}^+]:[\text{NADH}]$  ratio and lowering NAM levels. Blockade of either AMPK or Nampt counteracts the effects of GR, and, conversely, activation of AMPK, in normocaloric conditions, augments intracellular  $[\text{NAD}^+]:[\text{NADH}]$  ratio, reduces NAM levels, and mimics GR. Inhibition of cell differentiation induced by GR, AMPK activation, or Nampt is dependent on SIRT1 because skeletal myoblasts derived from *SIRT1*<sup>+/-</sup> heterozygous animals are less sensitive to either GR or AMPK activation and continue to differentiate in extremely low caloric conditions. These findings provide an initial description and mechanistic explanation of how mammalian skeletal muscle cells sense, decode, and respond to nutrient availability through a series of highly regulated enzymatic reactions leading to modifications of metabolic parameters consonant with promoting activation of SIRT1.

## RESULTS

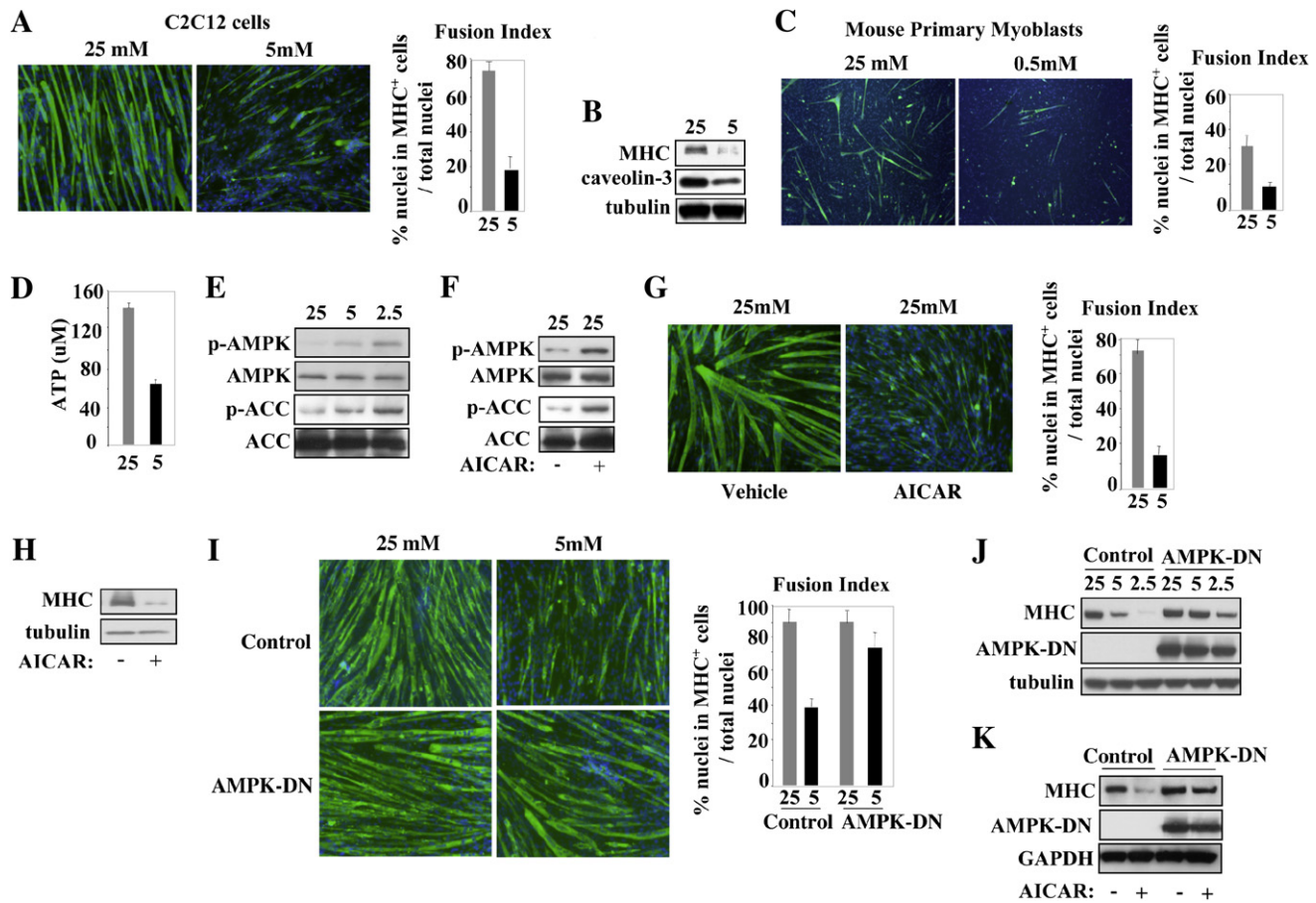
### Glucose-Restriction-Mediated Activation of AMPK Prevents Differentiation of Skeletal Muscle Cells

We investigated the effect of reducing the glucose levels—the major source of calories in the culture medium—on the differentiation process of either C2C12 skeletal muscle cell line or mouse primary skeletal myoblasts. Cells cultured in low glucose—5 mM or lower concentrations—failed to appropriately differentiate, as

indicated by reduced expression of the sarcomeric myosin heavy chain (MHC), caveolin-3, and impaired formation of multinucleated myotubes (Figures 1A–1C, see Figure S1A in the Supplemental Data available with this article online). Primary skeletal myoblasts differentiated in 5 mM glucose (data not shown), showing defective differentiation only at a lower glucose concentration (0.5 mM) (Figure 1C). Within the time frame of our experiments (48 hr in differentiation medium [DM]), GR did not induce apoptosis, and, once normocaloric conditions were re-established, cells resumed differentiation (Figures S1B and S1C). We evaluated whether fatty acids—which are effectively utilized by the mitochondrial metabolism—could overcome the effects of low glucose by exposing C2C12 cells to 0.1 mM oleic acid. Oleic acid promoted differentiation (Hurley et al., 2006) but was ineffective in counteracting the differentiation defects exerted by low glucose (Figure S1D), indicating that increased  $\beta$  oxidation fueled by lipids is insufficient to compensate for glucose reduction. As expected, cells cultured with low glucose had decreased intracellular ATP levels (Figure 1D). In response to ATP depletion, AMPK is phosphorylated and activated (Hardie et al., 2006). Accordingly, progressive reduction of glucose induced phosphorylation of AMPK and of its substrate acetyl-CoA-carboxylase (ACC) in C2C12 cells (Figure 1E). To evaluate whether AMPK activation is sufficient to recapitulate the effects of GR, we employed the AMP mimetic 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) (Kemp et al., 1999). AMPK is required for AICAR-stimulated glucose uptake in skeletal muscle, indicating that this AMP mimetic is a specific activator of AMPK in this tissue (Mu et al., 2001). AICAR promoted AMPK and ACC phosphorylation in normocaloric (NC) conditions (Figure 1F), and cells exposed to AICAR in NC conditions failed to appropriately differentiate (Figures 1G and 1H). In addition to AICAR, two other AMPK activators—the furancarboxylic acid derivative D942 (Kosaka et al., 2005) and the hypoglycemic drug metformin (Zhou et al., 2001)—also inhibited cell differentiation in a dose-dependent manner (Figures S1E and S1F). To test whether AMPK activation is necessary to mediate GR, we retrovirally transduced an AMPK dominant negative (AMPK-DN) construct—bearing the K45R mutation in the  $\alpha$ -2 catalytic subunit of rat AMPK (Mu et al., 2001)—in myoblasts. Cells that received the AMPK-DN efficiently differentiated despite the GR conditions (Figures 1I and 1J) and were refractory to AICAR-induced block of differentiation (Figure 1K). Moreover, compound C, an AMPK inhibitor (Zhou et al., 2001), also rescued the GR-induced differentiation defects of both C2C12 cells and primary skeletal myoblasts (Figures S1G and S1H). Thus, AMPK activation is required to mediate the effects of GR on skeletal muscle differentiation.

### Glucose Restriction and AMPK Require SIRT1

Because the SIRT1 ortholog Sir2 mediates the effects of calorie restriction in yeast (Lin et al., 2000) and counteracts skeletal myogenesis in mammalian cells (Fulco et al., 2003), we evaluated the potential involvement of SIRT1 in mediating the effects of GR on skeletal muscle differentiation. NAM—a sirtuin inhibitor (Bitterman et al., 2002)—rescued differentiation of GR cells (Figure 2A), suggesting that the deacetylase activity of sirtuins is relevant in mediating the effects of CR. NAM inhibits the deacetylase activities of several sirtuins. Therefore, we assessed



**Figure 1. Glucose Restriction Inhibits Differentiation of Skeletal Myoblasts and Activates AMPK**

(A) C2C12 cells were differentiated in a medium (differentiation medium [DM]) supplemented with either 25 mM or 5 mM glucose for 48 hr. Immunofluorescence (IF) was performed with a MHC antibody. DAPI marks the cell nuclei. The fusion index reported throughout this study was determined as described in the [Experimental Procedures](#) ( $p < 0.05$ ).

(B) Immunoblot for MHC, caveolin-3, and tubulin proteins from cell extracts derived from C2C12 cells cultured as in (A).

(C) Mouse primary myoblasts were cultured in DM with either 25 mM or 0.5 mM glucose for 48 hr. IF was performed as in (A).

(D) The ATP levels were determined in extracts of C2C12 cells cultured in DM with either 25 mM or 5 mM glucose.

(E) Phosphorylation of AMPK (pT172) and of the AMPK substrate ACC (pS79) in C2C12 cells cultured in DM with 25 mM, 5 mM, or 2.5 mM glucose determined by immunoblot with phospho-specific AMPK and ACC antibodies. The total levels of AMPK and ACC were determined with AMPK and ACC antibodies.

(F) C2C12 cells were exposed to AICAR (0.5 mM), and AMPK and ACC phosphorylation was evaluated as in (E).

(G) C2C12 cells were cultured in DM with 25 mM glucose and exposed to either vehicle (DMSO) or AICAR (0.5 mM) for 36 hr. IF was performed with an MHC antibody.

(H) MHC and tubulin immunoblot of extracts of the cells described in (G).

(I) C2C12 cells transduced with a retrovirus expressing a dominant negative form of AMPK (myc-tagged AMPK-DN) were cultured in DM supplemented with either 25 mM or 5 mM glucose. IF was performed with an MHC antibody.

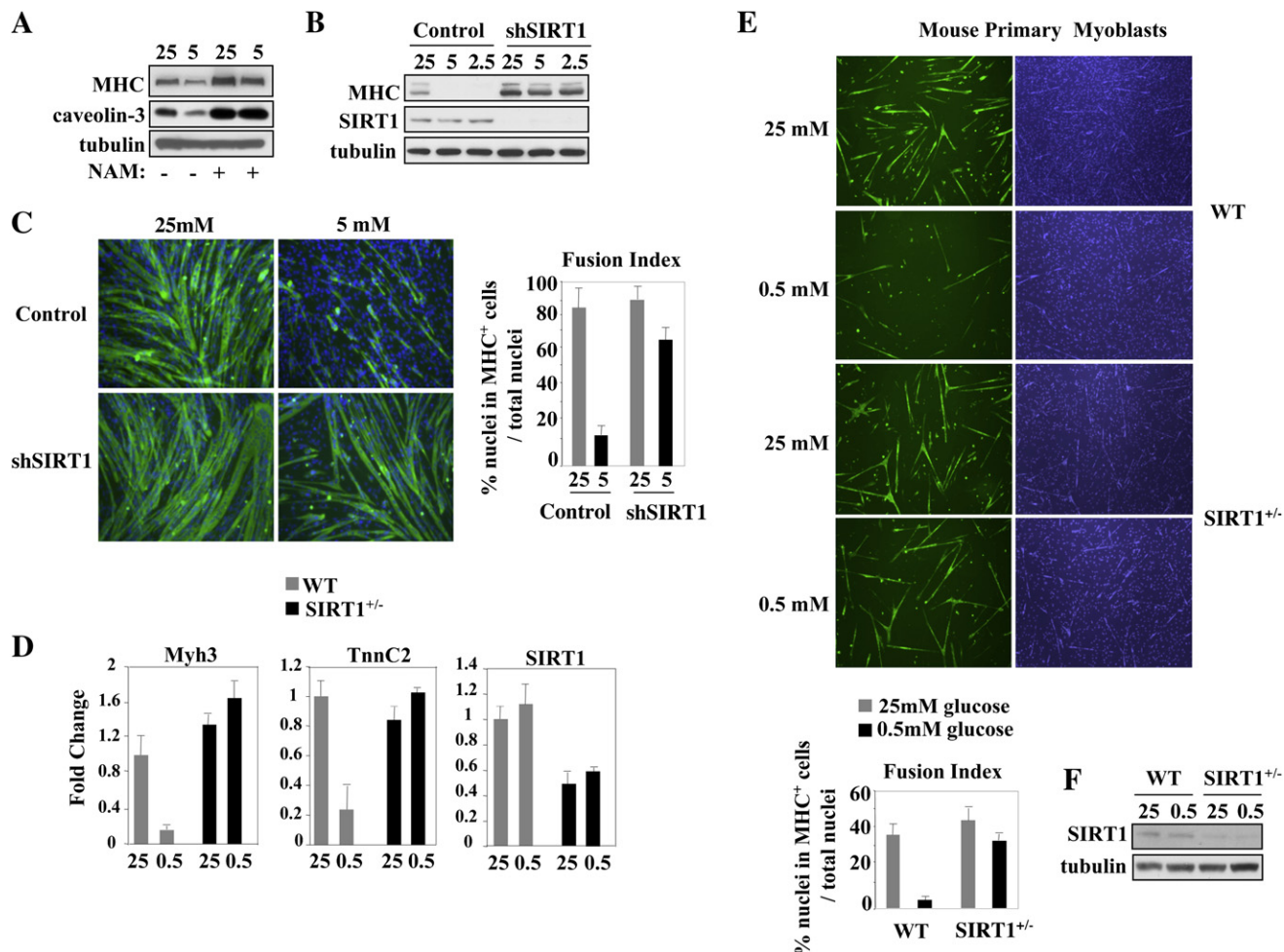
(J) Immunoblot of extracts derived from the cells described in (I) with the MHC, myc, and tubulin antibodies.

(K) MHC, myc, and GAPDH immunoblot of extracts derived from the cells expressing myc-AMPK-DN exposed to AICAR (0.5 mM).

the specific role of SIRT1 in GR by reducing its levels with a retrovirus expressing a short hairpin RNA predicted to target solely the SIRT1 messenger RNA (mRNA) (shSIRT1). Under these conditions, the differentiation ability was efficiently rescued, even when the cells were cultured in very low glucose conditions (2.5 mM glucose) ([Figures 2B and 2C](#)). In contrast, small interfering RNA (siRNA)-mediated knockdown of two other mitochondrial sirtuins—SIRT3 and SIRT4—was ineffective in preventing GR-mediated inhibition of cell differentiation ([Figure S2](#)). To unequivocally test for SIRT1 involvement, we isolated skeletal myoblasts derived from mice with germline mutation of the *SIRT1*

gene. Because *SIRT1*<sup>-/-</sup> homozygous mice are perinatal lethal ([McBurney et al., 2003](#)), we compared the response to GR of primary myoblasts isolated from 4-week-old wild-type and *SIRT1*<sup>+/-</sup> heterozygous mice. Despite the extreme GR regimen (0.5 mM glucose), *SIRT1*<sup>+/-</sup> primary myoblasts efficiently activated muscle gene expression and differentiated, whereas the cells derived from wild-type littermates were impaired in these processes ([Figures 2D and 2E](#)). Neither the SIRT1 transcripts nor the protein levels were affected by GR in C2C12 cells, WT, or *SIRT1*<sup>+/-</sup> myoblasts ([Figures 2B, 2D, and 2F](#)). Thus, myoblasts cultured in low glucose are impaired in their differentiation





**Figure 2. SIRT1 Mediates the Effects of Glucose Restriction on Skeletal Myoblasts**

(A) Extracts of C2C12 cells cultured in DM with either 25 mM or 5 mM glucose and without or with NAM (5 mM) were immunoblotted with the MHC, caveolin-3, and tubulin antibodies.

(B) C2C12 cells were transduced with a retrovirus expressing a RNA hairpin directed against SIRT1 (shSIRT1) and cultured in DM with 25 mM, 5 mM, or 2.5 mM glucose. Immunoblot was performed with antibodies against MHC, SIRT1, and tubulin.

(C) MHC IF of cells expressing control or hSIRT1 cultured in DM with 25 mM or 5 mM glucose.

(D) RT-qPCR of transcripts (embryonic myosin heavy chain Myh3, troponin C2, and SIRT1) derived from freshly isolated mouse primary skeletal myoblasts of 4-week-old wild-type (WT) or SIRT1<sup>+/-</sup> heterozygous mice cultured in DM with 25 mM or 0.5 mM glucose. Three independently obtained RNA samples were evaluated. The values of each transcript were corrected for those of GAPDH transcripts. Error bars represent standard deviations ( $p < 0.05$ ).

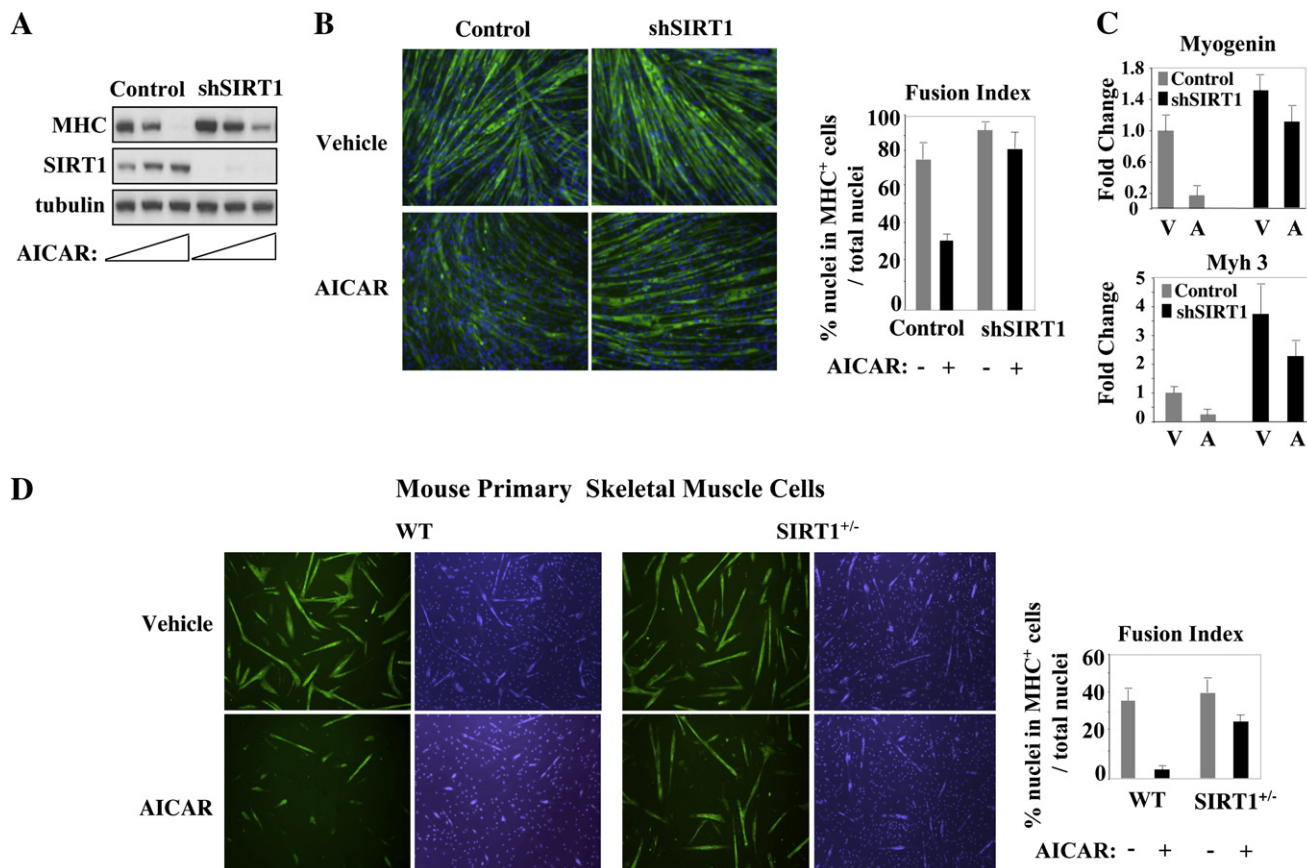
(E) MHC IF of WT and SIRT1<sup>+/-</sup> skeletal myoblasts cultured in DM with 25 mM or 0.5 mM glucose.

(F) SIRT1 and tubulin immunoblot of extracts from mouse primary myoblasts obtained from either WT or SIRT1<sup>+/-</sup> heterozygous animals cultured in either 25 mM or 0.5 mM glucose.

process and SIRT1 is required to mediate this phenomenon. We then asked whether AMPK also requires SIRT1. When SIRT1 levels were reduced, the cells became partially refractory to AICAR (Figures 3A–3C). Similarly, myoblasts from SIRT1<sup>+/-</sup> animals differentiated despite the presence of AICAR in the culture medium (Figure 3D). The residual inhibitory effect of AICAR on cell differentiation (on either shSIRT1 C2C12 cells or SIRT1<sup>+/-</sup> myoblasts) is likely due to the remaining SIRT1. SIRT1 was also required for the effects of the AMPK activator D942 (Figure S1E). Overall, the results of the experiments reported in this paragraph indicate that the effects of either GR or AMPK on skeletal myogenesis require SIRT1.

### Modulation of Gene Expression by GR

To identify genes transcriptionally modulated by glucose, we performed whole-genome microarray analysis of C2C12 cells grown in either 25 mM or 5 mM glucose. In addition to transcripts for structural and regulatory muscle proteins, numerous others involved in glucose and lipid metabolism, xenobiotic detoxification, mitochondrial energy production, and respiration were modulated by GR (Gene Expression Omnibus database at <http://www.ncbi.nlm.nih.gov/geo/> and Table S1). Expression of several transcripts was verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (data not shown and Figure S3). Downregulation of glycolysis is a hallmark of



**Figure 3. AMPK Activation Recapitulates the Effects of Glucose Restriction and Is SIRT1 Dependent**

(A) MHC, SIRT1, and tubulin immunoblot of extracts of C2C12 cells expressing shSIRT1 cultured in 25 mM glucose and exposed to increasing (0.25–0.5 mM) concentrations of AICAR for 36 hr.

(B and C) MHC IF (B) and myogenin and Myh3 transcripts (C) of cells described in (A). V, DMSO; A, AICAR. Error bars represent standard deviations.

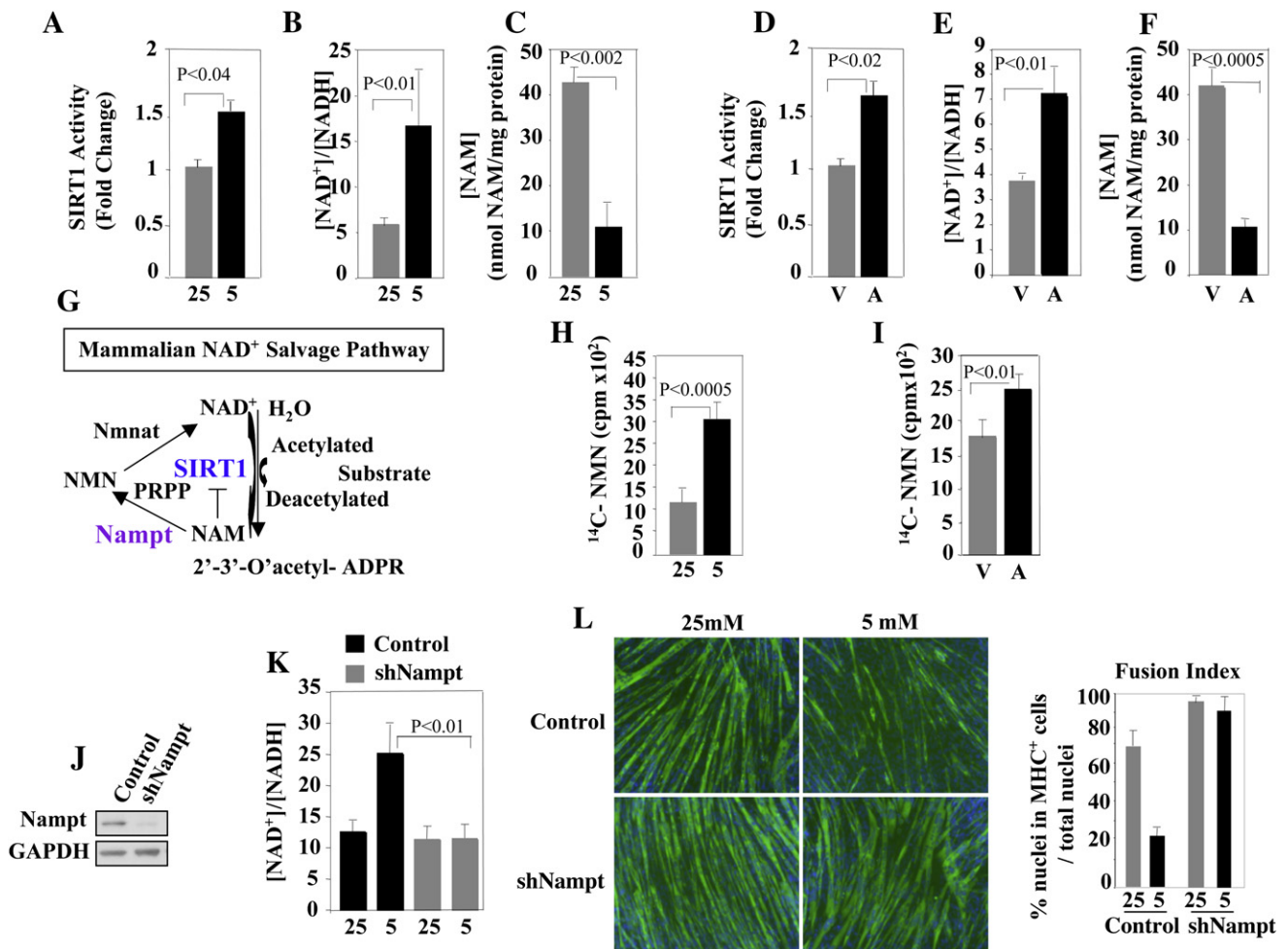
(D) MHC IF of mouse primary skeletal myoblasts from WT or SIRT1<sup>+/-</sup> heterozygous mice cultured in DM with DMSO (vehicle) or AICAR (0.5 mM) for 36–48 hr.

calorie restriction and it has been suggested as one of the mechanisms that mediates its effects (Ingram et al., 2006; Hipkiss, 2006). Accordingly, the transcripts of the glycolytic genes phosphoglycerate mutase (PGAM), phosphofructokinase, and beta enolase were reduced (Table S1 and Figures S3A and S3B). Expression of the phosphoglycerate mutase, beta enolase, and phosphofructokinase was also reduced in skeletal muscles of mice subjected to a 48 hr fasting (Figure S3B). Conversely, the UDP-glucuronosyltransferase 1, epoxide hydrolase 1 (Epx1) and glutathione S transferase (GST) (xenobiotic detoxification), and Gadd45 gamma (stress-induced) transcripts were increased in GR cells (Table S1 and Figure S3C). As observed in *C. elegans* subjected to dietary glucose restriction (Schulz et al., 2007), several transcripts encoding for proteins involved in lipid metabolism were increased, whereas a number of transcripts encoding collagen or collagen-like proteins were decreased in GR cells (Table S1, extracellular matrix). We next asked whether the modifications on gene expression induced by GR were dependent on SIRT1 by either exposing the GR cells to NAM or by overexpressing SIRT1 in NC conditions. The results of these experiments indicate that NAM reversed the effects of GR on gene expression and, conversely, SIRT1 mimicked them under NC (Figures S3A and S3C). The transcripts for the PGAM, GST,

and Epx1 genes were also evaluated in myoblasts from either wild-type or SIRT1<sup>+/-</sup> mice. Although GR affected their expression in control myoblasts, it had no effect on SIRT1<sup>+/-</sup> cells. (Figure S4). Overall, the results of these experiments indicate that GR induces specific modifications on the gene expression profile and that this gene modulation involves SIRT1.

#### The Nampt of the NAD<sup>+</sup> Salvage Pathway Mediates the Effects of GR or AMPK on Cell Differentiation in a SIRT1-Dependent Manner

Because the SIRT1 levels were not increased by GR, we considered the possibility that its enzymatic activity may be modulated. Indeed, extracts derived from GR cells sustained an increased SIRT1 activity (Figure 4A). SIRT1 activity is stimulated by an increased [NAD<sup>+</sup>]:[NADH] ratio and/or reduced NAM levels. Given that either GR or AMPK requires the presence of SIRT1 and its activity is increased in GR cells, we asked whether the [NAD<sup>+</sup>]:[NADH] ratio and NAM levels were influenced by GR or AMPK activation. Extracts derived from GR cells displayed a significantly increased [NAD<sup>+</sup>]:[NADH] ratio and decreased NAM (Figures 4B and 4C, Figure S5A). Similarly, AICAR increased SIRT1 activity and the [NAD<sup>+</sup>]:[NADH] ratio and decreased the NAM levels (Figures 4D–4F, Figure S5B). AICAR also stimulated SIRT1 activity in



**Figure 4. Effects of Glucose Restriction, AMPK Activation, or Nampt on  $[NAD^+]:[NADH]$  Ratio, SIRT1 Activity, and Nicotinamide Levels of Skeletal Muscle Cells**

(A) SIRT1 activity was evaluated in extracts derived from C2C12 cells cultured in DM with 25 mM or 5 mM glucose, respectively, for 24 hr.

(B and C)  $[NAD^+]:[NADH]$  ratio (B) and NAM levels (C) were determined in extracts of C2C12 cells cultured in DM with 25 mM or 5 mM glucose.

(D) SIRT1 activity was evaluated in extracts derived from C2C12 cells exposed to vehicle or AICAR (0.5 mM) for 24 hr.

(E and F)  $[NAD^+]:[NADH]$  ratio (E) and NAM levels (F) were evaluated in C2C12 cells cultured in DM with 25 mM glucose in the absence (vehicle, V) or presence (A) of AICAR.

(G) Schematic representation of the SIRT1-mediated deacetylation reaction and  $NAD^+$  salvage pathway.

(H) Nampt enzymatic activity in extracts of C2C12 cells cultured in DM with 25 mM or 5 mM glucose.

(I) Nampt enzymatic activity in extracts of C2C12 cells cultured in DM with 25 mM glucose in the absence (V) or presence (A) of AICAR.

(J) Nampt and GAPDH immunoblot of C2C12 cells expressing a RNA hairpin against Nampt (shNampt).

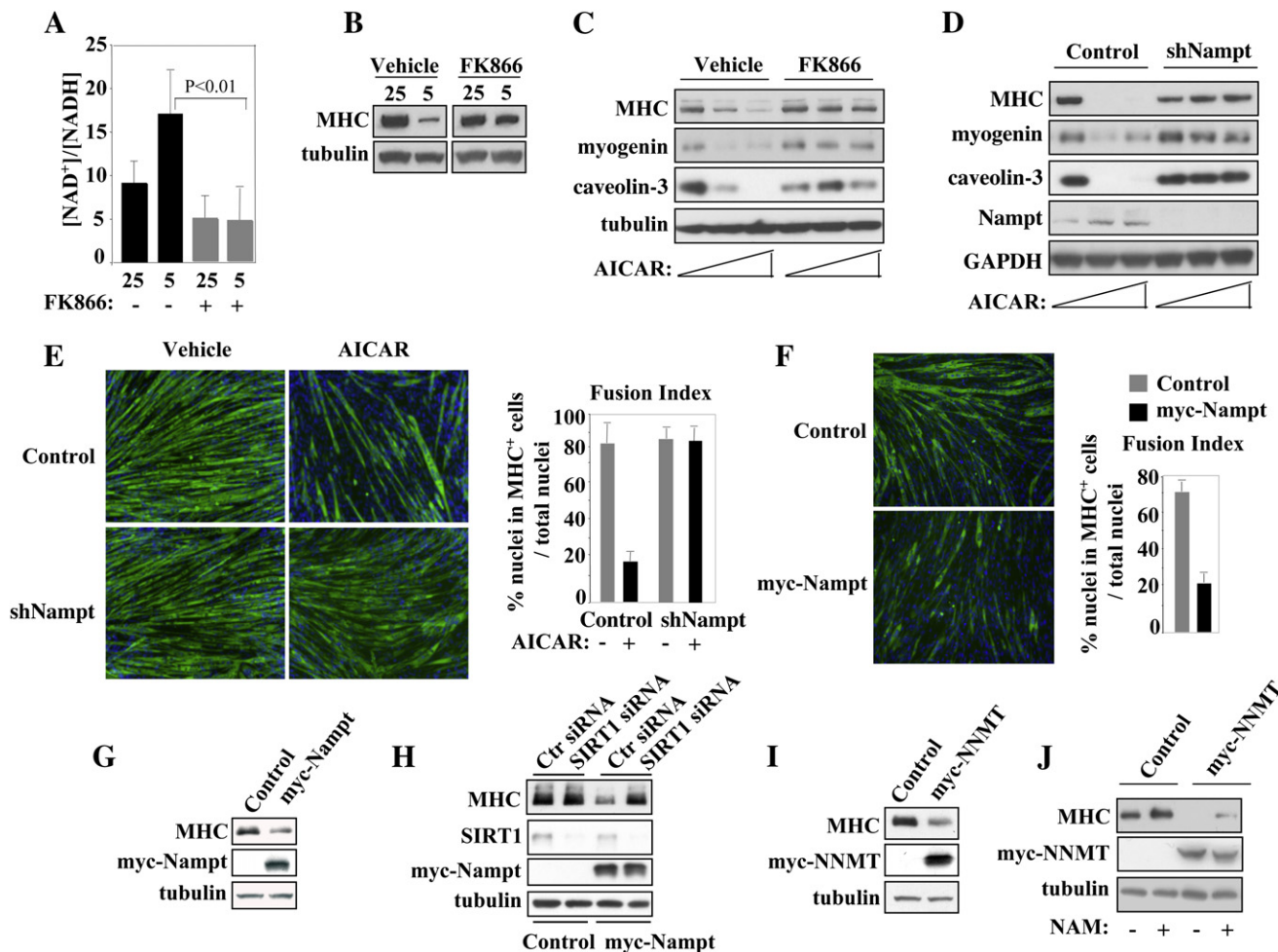
(K)  $[NAD^+]:[NADH]$  ratio in cells expressing shNampt cultured in DM with 25 mM or 5 mM glucose.

(L) MHC IF of control or shNampt-expressing C2C12 cells cultured in DM with 25 mM or 5 mM glucose. All of the enzymatic measurements reported in this figure were repeated with three independent samples. Error bars represent standard deviations.

wild-type mouse primary myoblasts and—consistent with the residual inhibitory effect of AICAR on cell differentiation (Figure 3D)—in *SIRT1*<sup>−/−</sup> myoblasts (Figure S4B). The increased intracellular  $[NAD^+]:[NADH]$  ratio and reduced NAM levels observed in GR- and AICAR-treated cells are consistent with activation of the  $NAD^+$  salvage pathway. In a highly regulated deacetylation reaction, SIRT1 cleaves  $NAD^+$ , yielding NAM, 2'-3'-O-acetyl ADP ribose, and the deacetylated lysine (Tanny and Moazed, 2001; Sauve et al., 2006). NAM is then employed as a precursor of  $NAD^+$  synthesis through the  $NAD^+$  salvage pathway. In mammalian cells, Nampt catalyzes the conversion of NAM and phosphor-

ibosyl pyrophosphate (PRPP) into nicotinamide mononucleotide (NMN) (Magni et al., 1999). NMN is further converted into  $NAD^+$  by the nicotinamide mononucleotide adenylyltransferase (Emanuelli et al., 2001; Figure 4G). Because Nampt is the first and rate-limiting enzyme of this pathway, we tested for its involvement in GR- and AMPK-mediated effects on skeletal myogenesis. To evaluate the Nampt enzymatic activity, we incubated cell extracts derived from skeletal muscle cells cultured in either NC or GR conditions with  $^{14}C$ -labeled NAM (the Nampt substrate) and measured formation of  $^{14}C$ -labeled NMN (the final product of the Nampt reaction). Cell extracts from either GR- or AICAR-treated





**Figure 5. The Outcomes of AMPK Activation on Cell Differentiation Depend on Nampt and SIRT1**

(A) [NAD<sup>+</sup>]/[NADH] ratio in C2C12 cells exposed to the Nampt inhibitor FK866 (10 nM) cultured in DM with either 25 mM or 5 mM glucose.  
 (B) MHC and tubulin immunoblot of extracts from C2C12 cells exposed to FK866 (10 nM).  
 (C) MHC, myogenin, caveolin-3, and tubulin immunoblot of extracts of C2C12 cells exposed to increasing concentrations (0.25–0.5 mM) of AICAR and FK866 (10 nM) in DM with 25 mM glucose.  
 (D) MHC, myogenin, caveolin-3, Nampt, and GAPDH immunoblot of extracts from C2C12 cells (control or shNampt) exposed to AICAR in DM with 25 mM glucose.  
 (E) MHC IF of control or shNampt-expressing C2C12 cells cultured in DM with 25 mM glucose and 0.5 mM AICAR or vehicle.  
 (F) MHC IF of controls or myc-Nampt-expressing C2C12 cells cultured in DM with 25 mM glucose.  
 (G) MHC, myc, and tubulin immunoblot of extracts from the C2C12 cells described in (F).  
 (H) MHC, SIRT1, myc, and tubulin immunoblot of extracts from C2C12 cells expressing myc-Nampt and transfected with either control (scrambled) or SIRT1 siRNA. Cells were cultured in DM with 25 mM glucose.  
 (I) MHC, myc, and tubulin immunoblot of extracts from C2C12 cells expressing myc-NNMT and cultured in DM with 25 mM glucose.  
 (J) Immunoblot were as described in (I) with extracts from control or NNMT-expressing C2C12 cells cultured in the absence or presence of NAM (5 mM).

cells sustained an increased production of <sup>14</sup>C-NMN, compared to extracts of NC cells (Figures 4H and 4I). The function of Nampt was directly addressed by lowering of its levels with a retrovirus expressing a short-hairpin-specific RNA (shNampt) (Figure 4J). Cells with reduced Nampt did not increase the intracellular [NAD<sup>+</sup>]/[NADH] ratio and efficiently differentiated in GR conditions (Figures 4K and 4L, and Figure S5C). We then probed the role of the enzymatic activity of Nampt with FK866, a highly specific inhibitor (Hasmann and Schemainda, 2003; Khan et al., 2006). FK866 prevented the increase of the intracellular [NAD<sup>+</sup>]/[NADH] ratio caused by GR and allowed differentiation of myoblasts cul-

tured in GR conditions (Figures 5A and 5B). To further substantiate these findings, cells were transduced with a Nampt mutant (A244M) that retains the phosphoribosyltransferase activity but is FK866 insensitive (Khan et al., 2006). Because the Nampt A244M protein escapes FK866 inhibition, these cells had an increased intracellular [NAD<sup>+</sup>]/[NADH] ratio and failed to appropriately differentiate, despite exposure to FK866 (Figures S6A and S6B, middle panels). The enzymatic activity of Nampt was inactivated by introduction of a mutation (S314A) within its active domain (Wang et al., 2006). Cells transduced with Nampt (A244M/S314A) and exposed to FK866 failed to upregulate the

[NAD<sup>+</sup>]:[NADH] ratio and properly differentiated (Figures S6A and S6B, right panels). Overall, these results indicate that the enzymatic activity of Nampt is responsible for modulating the [NAD<sup>+</sup>]:[NADH] ratio and is associated with lack of cell differentiation observed during GR. In parallel experiments, we employed AICAR to ask whether Nampt was also required to mediate the effects of AMPK. As shown in Figures 5C–5E and Figure S7, inhibition of Nampt activity with FK866 or reduction of the Nampt levels rendered the cells refractory to AICAR. Lastly, we investigated whether Nampt requires SIRT1. To this end, skeletal myoblasts were transduced with a retrovirus encoding Nampt. Under NC conditions, cells overexpressing Nampt were impaired in their differentiation process (Figures 5F and 5G). Reduction of SIRT1 levels resumed differentiation of Nampt-overexpressing cells, as indicated by increased MHC expression (Figure 5H, compare Ctr and SIRT1 siRNA lanes). In an attempt to distinguish the contribution of the two possible effects mediated by Nampt (NAM depletion versus increased NAD<sup>+</sup> synthesis), we transduced C2C12 cells with a retrovirus expressing the NAM N-methyltransferase (NNMT). NNMT is an enzyme outside of the NAD<sup>+</sup> salvage pathway that removes NAM by converting it to N-methyl-NAM without boosting NAD<sup>+</sup> biosynthesis (Aksoy et al., 1994; Anderson et al., 2003). Cells expressing NNMT were impaired in their differentiation despite being cultured with high glucose (Figure 5I). Consistent with a role of NAM in this process, its addition partially reverted the NNMT-mediated inhibition on differentiation (Figure 5J). Altogether, the results presented in this paragraph indicate that (1) GR and AMPK activate Nampt, (2) Nampt is required for the effects exerted by GR and AMPK, (3) the effects of Nampt on cell differentiation require SIRT1, and (4) expression of NNMT—which reduces NAM levels (Aksoy et al., 1994)—is sufficient to mimic GR.

#### Fasting Induces Expression of Some AMPK Target Genes in a SIRT1-Dependent Manner

So that the role of AMPK-SIRT1 in regulating the response to nutrient availability in vivo could be evaluated, wild-type CD1 or *SIRT1*<sup>−/−</sup> homozygous animals outbred on the CD1 strain were fed ad libitum (AL) or fasted for 48 hr. We chose to analyze *SIRT1*<sup>−/−</sup> homozygous/CD1 mice because these animals have no detectable SIRT1 and, in contrast to inbred *SIRT1*<sup>−/−</sup>/129/Sv, survive to adulthood (McBurney et al., 2003). Uncoupling protein 2 (UCP-2) expression is induced by AICAR (Jager et al., 2007) and is repressed by SIRT1 in basal conditions (Bordone et al., 2007; Moynihan et al., 2005). Compared to the fed-AL wild-type (Figures 6A and 6B, lanes 1 and 2), UCP-2 was increased in muscles of fed AL *SIRT1*<sup>−/−</sup> mice (Figures 6A and 6B, lanes 5 and 6). Although fasting induced UCP-2 expression in the wild-type (Figures 6A and 6B, lanes 3 and 4), it failed to further increase the already elevated levels of UCP-2 in *SIRT1*<sup>−/−</sup> animals (Figures 6A and 6B, lanes 7 and 8). A similar behavior was noted for expression of uncoupling protein 3 (UCP-3)—another gene whose expression is induced by AICAR (Jager et al., 2007) and repressed by SIRT1 (Amat et al., 2007; Figures 6A and 6B) and for the pyruvate kinase-4 (PK4) (Figure 6A). In contrast, expression of the fatty acid translocase CD36—a gene activated by AICAR (Chabowski et al., 2006)—was stimulated by fasting, irrespective of SIRT1 (Figure 6A). Consistent with a repressive role of SIRT1 on muscle gene expression (Fulco et al., 2003), skeletal muscles

of *SIRT1*<sup>−/−</sup> had elevated levels of perinatal and embryonic myosins—two isoforms that are normally repressed in the adult muscle (Weydert et al., 1987). On the contrary, the nonmuscle myosin Myl6—whose expression is not developmentally regulated—was comparably expressed in wild-type and *SIRT1*<sup>−/−</sup> mice (Figures 6C and 6D). Because the levels of Pax7, Myf5, cyclins D1 and E, and PCNA—all markers of quiescent or activated and proliferating satellite cells (Holterman and Rudnicki, 2005)—were not increased (Figures S7C and S7D), we are inclined to exclude the possibility that elevated expression of perinatal and embryonic myosins is a reflection of muscle damage and regeneration occurring in *SIRT1*<sup>−/−</sup> animals. Overall, the results presented in this paragraph indicate that SIRT1 negatively regulates expression of some AMPK targets in normocaloric conditions in vivo and is required to mediate their induction by fasting. They further indicate that skeletal muscles of adult *SIRT1*<sup>−/−</sup> mice retain expression of embryonic and neonatal differentiation markers without displaying signs of satellite cell activation.

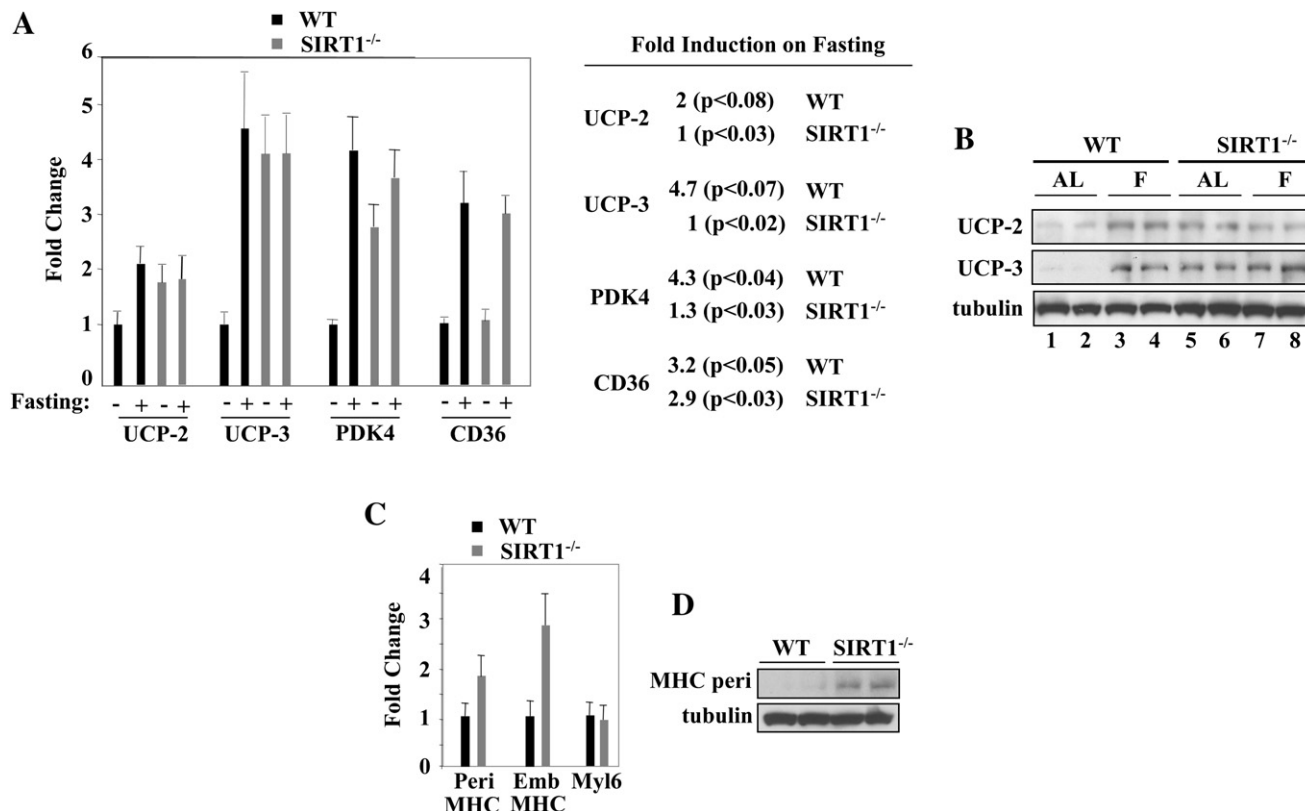
#### Glucose Restriction Induces Nampt Transcription via AMPK

Extracts derived from either GR- or AICAR-treated cells have an elevated Nampt enzymatic activity (Figures 4H and 4I). Therefore, we asked whether these two interventions might influence Nampt transcription. After GR of skeletal muscle cells or animal fasting, the Nampt mRNA and protein levels were increased (Figures 7A–7C). Cells exposed to AICAR had also increased Nampt mRNA and protein levels (Figures 7D and 7E). Such increase in Nampt was blunted in cells cultured in GR conditions and expressing an AMPK-dominant negative (AMPK-DN) form (Figure 7F), indicating that AMPK regulates GR-induced expression of Nampt. Consonant with a lack of Nampt induction, neither the Nampt activity nor the [NAD<sup>+</sup>]:[NADH] ratio was increased in AMPK-DN cells exposed to GR (Figures 7G and 7H). Overall, these experiments indicate that activated AMPK is required to mediate transcriptional induction of Nampt occurring during GR.

#### DISCUSSION

SIRT1 regulates skeletal muscle differentiation in a tissue culture system (Fulco et al., 2003), but how signals emanating from the microenvironment control this process is not understood. More specifically, whether and how modifications of the microenvironment may impact the intracellular NAD<sup>+</sup> biosynthesis and/or the nicotinamide levels—and therefore modulate SIRT1 activity—in skeletal muscle cells remains to be determined. The results of this study indicate that skeletal myoblasts cultured in low-glucose media are impaired in their differentiation through activation of a pathway that targets the enzymatic activity of SIRT1. The lack of proper differentiation when nutrients are scarce may be simplistically interpreted as the result of passive adaptation of cells incapable of sustaining energy-demanding processes, such as those associated with modification of gene expression, sarcomere assembly, and reorganization of the Golgi apparatus, that accompany differentiation. However, the results of our experiments suggest an alternative interpretation of the phenomenon and rather indicate the existence of a defined pathway that actively controls cell behavior in response to low nutrients. AMPK, Nampt, and SIRT1 are the molecular components of





**Figure 6. In Vivo Role of SIRT1 during Fasting**

(A) RT-qPCR of UCP-2, UCP-3, PDK4, and CD36 transcripts from hindlimb muscles of WT and *SIRT1*<sup>-/-</sup> mice fed ad libitum (AL) or fasted for 48 hr. The values of each transcript were corrected for those of GAPDH transcripts. Error bars represent standard deviations. The RNAs obtained from three (n = 3) animals per experimental group were analyzed. n-fold induction and p values are indicated in the right panel.

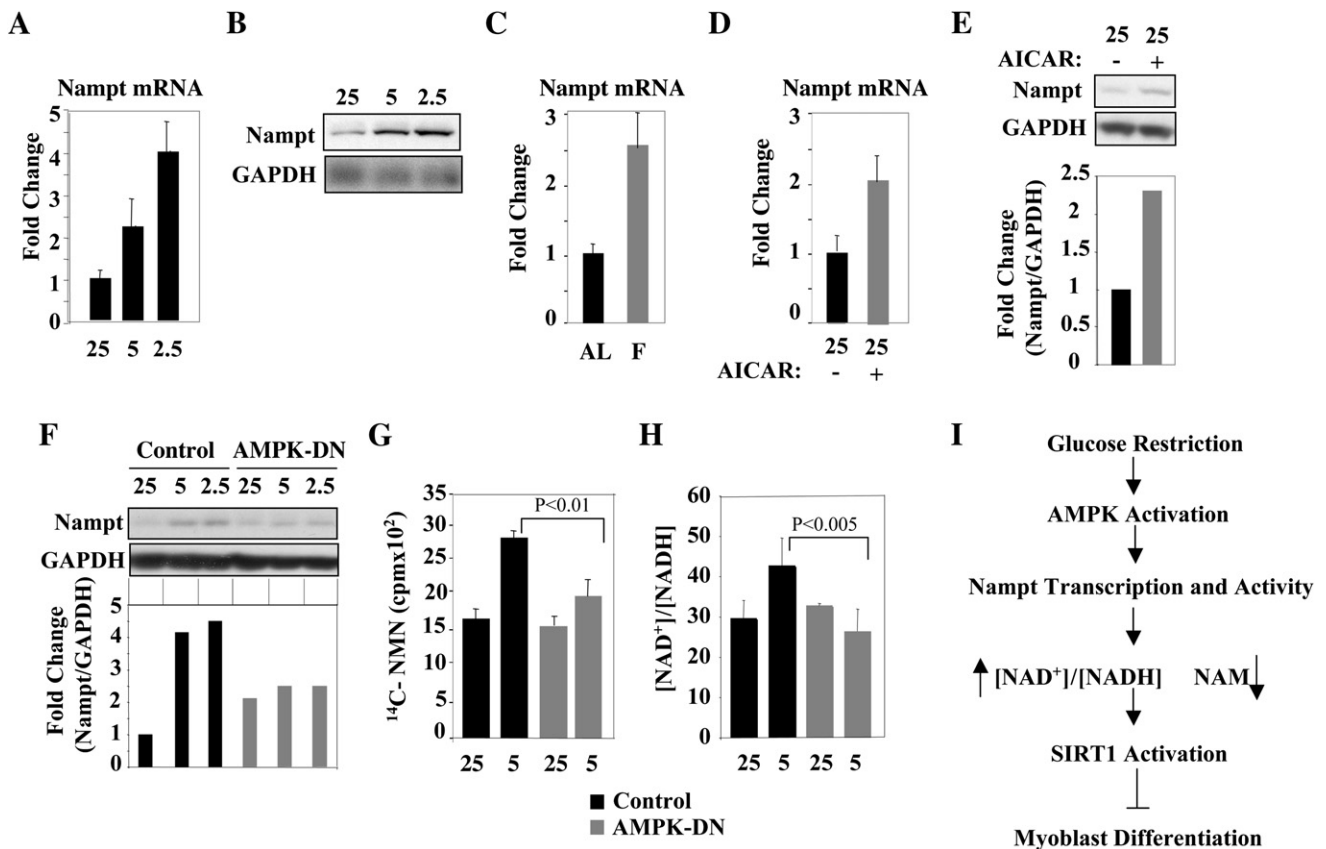
(B) UCP-2, UCP-3, and tubulin immunoblot of extracts derived from hindlimb muscles of WT mice fed AL (lanes 1 and 2) or fasted (F) for 48 hr (lanes 3 and 4) and *SIRT1*<sup>-/-</sup> mice fed AL (lanes 5 and 6) or fasted (F) for 48 hr (lanes 7 and 8). Muscles derived from two WT and two *SIRT1*<sup>-/-</sup> animals for each experimental condition were analyzed.

(C) RT-qPCR of embryonic Myh3 and perinatal Myh8, as well as nonmuscle Myl6 myosin transcripts in WT and *SIRT1*<sup>-/-</sup> mice. The values of each transcript were corrected for those of GAPDH transcripts. Error bars represent standard deviations. The RNAs obtained from three (n = 3) animals per experimental group were analyzed.

(D) MHC perinatal and tubulin immunoblot of extracts derived from hindlimb muscles of WT and *SIRT1*<sup>-/-</sup> mice. Two WT and two *SIRT1*<sup>-/-</sup> animals were analyzed.

this pathway, and each of them is required for the cell to respond to GR. Pharmacological inhibition, RNA interference, or hemizygosity of the single molecular components render skeletal muscle cells oblivious to the calorie-poor microenvironment, allowing them to differentiate in an otherwise nonpermissive microenvironment. As such, the AMPK-Nampt-SIRT1 pathway can be viewed as a checkpoint that allows the cell to sense and respond to a scarcity of nutrients in the immediate surroundings (Figure 7I). Experiments conducted in fasting mice revealed that SIRT1 mediates response of some AMPK target genes to nutrient availability. Because the contribution of satellite cells to physiological muscle fiber growth and maintenance in the adult animal is small (Spalding et al., 2005), it is likely that AMPK and SIRT1 may be differently activated and mediate distinct outcomes in cultured muscle cells exposed to low glucose and in muscles of an adult fasting animal. Central to the regulation of SIRT1 in cultured cells and to the functional consequences of GR on cell differentiation is the modulation of the intracellular [NAD<sup>+</sup>]:[NADH] ratio and the NAM levels engendered by GR. These two metabolic parameters

are regulated by the Nampt enzyme of the NAD<sup>+</sup> salvage pathway and are essential to convey cellular response induced by GR. The likelihood that free cellular NAD<sup>+</sup> is regulatory for SIRT1 activity is suggested by the estimated concentration of free nuclear NAD<sup>+</sup>, which approximates K<sub>m</sub> values of several sirtuins (Bedalov et al., 2003). Nonetheless, the elevated cytosolic free [NAD<sup>+</sup>]:[NADH] ratio (Williamson et al., 1967) makes it unlikely that the enzymatic activity of SIRT1 is modulated by a generalized increase of the NAD<sup>+</sup>. Compartmentalization of the NAD<sup>+</sup> biosynthesis (Yang et al., 2006) may create local gradients—in defined nuclear chromatin domains or organelles (Yang et al., 2007)—of NAD<sup>+</sup>, [NAD<sup>+</sup>]:[NADH] ratio, and NAM levels that could effectively regulate SIRT1 and other sirtuins. Indeed, reduction of the NAM levels obtained via expression of the NNMT in skeletal myoblasts is sufficient to recapitulate the effects of GR on cell differentiation. Although these findings do not exclude the possibility that the [NAD<sup>+</sup>]:[NADH] ratio may be functionally relevant, they further underscore the important role that NAM exerts in regulating phenomena controlled by SIRT1 (Anderson et al., 2003).



**Figure 7. AMPK Mediates the Glucose-Restriction-Induced Nampt Transcription**

(A) RT-qPCR of Nampt RNA transcripts from C2C12 cells cultured in DM with 25 mM, 5 mM, or 2.5 mM glucose. In all the experiments reported in this figure, the Nampt values were corrected for those of GAPDH transcripts. Error bars represent standard deviations.

(B) Nampt and GAPDH immunoblot of extracts from C2C12 cells cultured as described in (A).

(C) RT-qPCR of Nampt RNA transcripts from hindlimb muscles of mice (n = 3) subjected to a 48 hr fast (F) or fed AL.

(D) Nampt transcripts from C2C12 cells exposed to AICAR in DM with 25 mM glucose.

(E) Nampt immunoblot of extracts from C2C12 cells exposed to AICAR in DM with 25 mM glucose. Quantification was performed by scanning of the bands corresponding to the Nampt signal and correction of the values for those obtained from the GAPDH signal (Nampt:GAPDH ratio).

(F) Nampt and GAPDH immunoblot of extracts from C2C12 cells expressing AMPK-DN in DM with 25 mM, 5 mM, or 2.5 mM glucose.

(G) Nampt activity in extracts from control or AMPK-DN-expressing C2C12 cells cultured in DM with 25 mM or 5 mM glucose.

(H)  $[\text{NAD}^+]/[\text{NADH}]$  ratio in control or AMPK-DN-expressing C2C12 cells cultured in DM with 25 mM or 5 mM glucose.

(I) Schematic illustration of the GR-AMPK-Nampt-SIRT1 pathway.

Nampt expression is increased after several stress stimuli (Yang et al., 2007). Our experiments provide evidence that AMPK is involved in this regulation. AMPK activation—induced by GR or AICAR—causes the Nampt transcripts to increase in normocaloric conditions, whereas blockade of AMPK obtained with an AMPK dominant negative form prevents GR-mediated increased expression of Nampt. Intriguingly, a genomic region directing Nampt expression encompasses several putative binding sites for the forkhead FOXO transcription factors (M.F. and V.S., unpublished data), which are phosphorylated and activated by AMPK (Greer et al., 2007b). In addition to intracellular Nampt, a secreted form of Nampt has been described that reportedly exhibits robust  $\text{NAD}^+$  biosynthetic activity. Importantly, *Nampt*<sup>+/-</sup> heterozygous mice show impaired glucose tolerance and reduced glucose-stimulated insulin secretion (Revollo et al., 2007). Modification of the intracellular  $[\text{NAD}^+]/[\text{NADH}]$  ratio and of NAM levels are necessary but not sufficient to mediate the ef-

fects of GR and require the enzymatic activity of SIRT1. Increased expression (Cohen et al., 2004; Nemoto et al., 2004) coupled to increased SIRT1 activity—as we report here in GR skeletal muscle cells—may be part of a functionally coherent strategy developed by the cell to cope with reduced nutrient availability. It seems that increased Nampt expression controls different cellular outcomes depending on the location of the target sirtuins. In the case of genotoxic-stress-mediated cell death, the protective role of Nampt is exerted through the mitochondrial SIRT3 and SIRT4 (Yang et al., 2006), whereas GR-induced Nampt expression mediates its effects on the differentiation process of skeletal muscle cells through the nuclear SIRT1. Whether AMPK has a role also in the SIRT3-SIRT4-regulated response to genotoxic stress awaits further investigation.

There is little doubt that calorie restriction has several beneficial outcomes in adult organisms, especially in humans with a typical Western diet (Hursting et al., 2003). For instance,

reduction of calorie intake improves numerous functional indexes and reduces metabolic risks associated with type II diabetes and metabolic syndrome. Intriguingly, alternate-day fasting (ADF)—a regimen involving 1 day of ad libitum diet followed by 1 day of food withholding or reduction—has also been shown, though not conclusively, to improve insulin sensitivity, reduce blood pressure, and increase muscle fat oxidation (Varady and Hellerstein, 2007). Our study raises the possibility that AMPK-Nampt-SIRT1 may be the molecular pathway activated by calorie restriction or ADF regimens and by the hypoglycemic agent metformin, a drug commonly used to treat type II diabetes (Krentz and Bailey, 2005). Less investigated are the effects of reduced caloric intake during embryonic and fetal development, although it is well known that undernourishment is detrimental for fetal development with lasting consequences in adulthood. Earlier studies have shown that the skeletal muscle fiber numbers in young mammals (Bedi et al., 1982)—including humans (Montgomery, 1962)—are reduced after undernutrition of the mother during gestation and lactation. Interestingly, the mononucleated cells lying within the basal lamina (satellite cells) were not reduced, but the secondary myotubes were, indicating that the rate of myoblast differentiation (or proliferation) was impaired in the muscles of the undernourished animals (Wilson et al., 1988). Restoration of normal dietary intake could correct—within a given time frame—the muscle fibers defect of undernourished animals (Wilson et al., 1988). We speculate that, functioning as a cellular checkpoint, the AMPK-Nampt-SIRT1 pathway may be activated by reduced nutrient availability to prevent the undertaking of energy-demanding processes—such as cell differentiation—during calorie-unfavorable conditions and be inactivated, once nutrients become available, to allow resumption of physiological development.

## EXPERIMENTAL PROCEDURES

### Cell Culture, Small Molecules, and Retroviral Infection

C2C12 cells were obtained from American Type Culture Collection (ATCC) and grown in complete Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 20% fetal bovine serum (FBS) (growth medium [GM]) and 100  $\mu$ g/ml penicillin and streptomycin. When C2C12 were nearly confluent (60%–70% confluency), they were induced to differentiate in DMEM supplemented with 2% horse serum, insulin, transferrin, and selenium (1 $\times$ ) (Invitrogen) (differentiation medium [DM]) either in normocaloric (25 mM glucose) or reduced glucose conditions, as indicated in the single experiments. The fusion index was determined by counting of the number of nuclei present in MHC<sup>+</sup> cells per number of total nuclei and expressed as percentage as described in (Iezzi et al., 2004). Nuclei present in at least five microscopic fields were counted. NAM and metformin were obtained from Sigma. AICAR and D942, compound C, and the oleic acid were obtained from Calbiochem. The Nampt-specific inhibitor FK866 was kindly synthesized by the National Institute of Mental Health (NIMH) Chemical Synthesis and Drug Supply Program at the National Institutes of Health (NIH). Retroviruses were generated by transient transfection of retroviral constructs in Phoenix cells. The viral supernatants were employed to transduce C2C12 cells. Cells were selected with 2  $\mu$ g/ml puromycin.

### Antibodies, Immunoblotting, and Immunofluorescence

The methods and antibodies employed are reported in the Supplemental Data.

### Plasmids, siRNAs, and Retroviral Constructs

The constructs and siRNAs source are described in the Supplemental Data.

### Expression Profiling and Data Analysis

The methods are described in the Supplemental Data. A comprehensive annotation of the transcripts identified by genome-wide expression profiling has been deposited in the GEO database. The accession number is reported in the Supplemental Data.

### RT-qPCR

The methods and the oligonucleotide sequences are reported in the Supplemental Data.

### Mouse Primary Myoblasts

Skeletal primary myoblasts from 4-week-old wild-type or *SIRT1*<sup>+/-</sup>129/Sv mice were isolated and cultured as described in (Carette et al., 2004). To assay for the effects of GR on muscle differentiation, cells were plated on gelatin-coated tissue culture dishes and induced to differentiate in DM supplemented with either 25 mM or 0.5 mM glucose.

### Nampt Enzymatic Activity, NAD<sup>+</sup> and NADH Assays, and HPLC/MALDI/MS Determination of NAM

Detailed protocols for determining Nampt activity, NAD<sup>+</sup> and NADH levels, and HPLC/MALDI/MS evaluation of nicotinamide are reported in the Supplemental Data.

### SIRT1 Deacetylase Activity

SIRT activity was measured with the SIRT1 Fluorimetric Drug Discovery Kit (Biomol) according to the manufacturer's instructions with minor modifications as described in the Supplemental Data.

### ATP Measurements

ATP levels were determined as described in the Supplemental Data.

### Animal Studies

Six-week-old Balb/c mice were obtained from the Jackson Laboratory and housed at the animal vivarium of the NIAMS at the NIH. The control group ( $n = 3$ ) was fed AL with a standard diet (Harland Tekland extruded diet 2018, 57.26% carbohydrate), whereas the experimental group ( $n = 3$ ) was fasted for 48 hr before being euthanized. Five-month-old wild-type/CD1 or *SIRT1*<sup>+/-</sup> homozygous/CD1 mice (McBurney et al., 2003) ( $n = 3$  for each group) were fed AL or fasted as described for the Balb/c mice. Water was available to both the control and fasted animals at all times. The hindlimb muscles from both control and experimental animals were isolated and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA or protein extraction. Four-week-old wild-type or *SIRT1*<sup>+/-</sup> heterozygous/129/Sv mice (McBurney et al., 2003) were euthanized, and the hindlimb muscles were isolated and immediately employed to obtain skeletal myoblast cultures. All the animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, seven figures, one table, and Supplemental References and are available at <http://www.developmentalcell.com/cgi/content/full/14/5/661/DC1/>.

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